sarcomere length (SL). In this study, we used time-resolved in situ FRET to monitor the effects of Ca^{2+}-occupancy, XB state, and SL on N-cTnC opening in skinned cardiac muscle fibers. FRET donor (AEDANS) and acceptor (DDPM) modified double-cysteine mutant cTnC(13C/51C)AEDANS-DDPM was reconstituted into skinned muscle fibers to examine the N domain of cTnC (N-cTnC) opening. To study the effect of SL on structural transitions of cTnC, we monitored the protein structural transitions at low and high [Ca^{2+}] and SL 1.8 and 2.2 µm. Mg^{2+}-ADP and sodium orthovanadate (Vi) were used to examine the effects of non-cyclng strong and weak XBs, respectively. We found that strongly bound XBs alter structural transitions of cardiac troponin only at 2.2 µm. On the other hand, Vi blunted the SL dependent opening of N-cTnC such that weak XBs have no effect on N-cTnC at either [Ca^{2+}] or SL. In addition, distance distribution analysis indicated that N-cTnC adopts four unique conformations associated with the four states of thin filament regulation, and that N-cTnC conformational equilibria are caused by cycling XBs. Based on our findings, we conclude that the observed dependence of myosin positive feedback regulation on SL is an important determinant of the Frank-Starling law of the heart.

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Ca^{2+}-Regulatory Function of the Inhibitory Peptide Region of Cardiac Troponin I is Aided by the C-Terminus of Cardiac Troponin T: Effects of FHC Mutations Ctni R145G and Ctnt R278C, Alone and in Combination, on Filament Sliding

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Investigations of cardiomyopathy mutations in Ca^{2+} regulatory proteins troponin and tropomyosin provide crucial information about cardiac disease mechanisms, and provide insights into functional domains in the affected polypeptides. Hypertrophic cardiomyopathy-associated mutations TnI R145G, located within the inhibitory peptide (I_p) of human cardiac troponin I (hcTnI), and TnT R278C, located immediately C-terminal to the IT arm in human cardiac troponin T (hcTnT), share remarkable features: structurally, biochemically, and pathologically. Using bioinformatics, we find compelling evidence that affected regions of hcTnI and hcTnT, may be related_not just structurally_but also evolutionarily. Because alignment of TnI and TnT coincides with the known structure of the IT-arm, and both Arg mutations are located close to the C-terminal end of the IT-arm, we investigated functional relationships between hcTnI R145G and hcTnT R278C. We hypothesized that if the mutations affected function independently, then their effects would be additive in a double mutant complex. We characterized Tn complexes containing either mutation alone, or both mutations simultaneously, using in vitro motility assays run with varying [Ca^{2+}], temperature, or HMM density. Our most significant findings show that TnT R278C "rescued" some deleterious effects of TnI R145G at high Ca^{2+}, but exacerbated the loss of function (i.e., switching off the actomyosin interaction) at low Ca^{2+}. Taken together, our results raise the likelihood that cTnI's I_p sequence might share a common evolutionary origin with, and thus be structurally and functionally related to, the C-terminus of cTnT. In accord with this prediction, our experimental results suggest that the C-terminus of cTnT aids Ca^{2+}-regulatory function of cTnI I_p within the troponin complex.

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Impact of Troponin-I Phosphorylation on Human Cardiac Myofilament Function

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A long-term goal of work in our laboratories is to understand the structural basis of myofilament function, i.e. cross-bridge dynamics (CD) and myofilament length dependent activation (LDA) in striated muscle in heath and disease. Moreover, in cardiac muscle LDA underlies the Frank Starling Law of the Heart. Troponin phosphorylation, and in particular troponin-I (cTnI), has been suggested to be a pivotal modulator of myofilament function. Here we examined the impact of phosphorylation of distinct cTnI domains on CD and LDA in isolated human myocardium. Site specific phosphorylation was accomplished by charge mutation of hcTnI phosphomimics on the PKA (S23/24D), PKC (S42/44E; T143E), AMPK (S150D), and novel (S5/6D) sites, followed by recombinant protein exchange into skinned non-failing human LV cardiac muscle strips (~2mm long, and ~150um diameter. Force and ATPase activity was measured as function of [Ca^{2+}] at short and long sarcomere length (SL=2.0&2.3um). We found, LDA: hcTnI-S150D attenuated, hcTnI-S42/43E increase, and no effect for the other sites. CD as indexed by tension cost was: decreased for cTnI-S42/44E and hcTnI-S150D, and no effect for the other sites. We conclude that cTnI phosphorylation at distinct sites differentially affect cross-bridge cycling and length dependent activation in human myocardium. Structural analysis employing x-ray diffraction is underway to determine the structural basis for these phenomena. Supported by NIH HL075494, HL62426, GM103622.

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The R144W Mutation in Mouse Cardiac Troponin T Attenuates Cross-bridge Recruitment and Detachment Kinetics

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A missense mutation, R141W, in the strong tropomyosin-binding region of human cardiac troponin T (cTnT) is associated with dilated cardiomyopathy (DCM). Previous studies of steady-state contractile function suggest that DCM-related mutations in cTnT attenuate myofilament Ca^{2+} sensitivity. Steady-state observations by themselves may not be sufficient enough to provide a reliable link between different mutations and divergent cardiac phenotypes, especially at submaximal Ca^{2+} levels. It is now widely appreciated that dynamic relationships - rather than steady-state aspects of the force-pCa relationship - dominate in conditions under which cardiac muscle functions. To understand the effects of the R141W mutation on cardiac contractile dynamics, we created a mouse cTnT analog ($McTnT_{R144W}$) of the human mutation, R141W. $McTnT_{R144W}$ and the wild-type McTnT were individually reconstituted into detergent-skinned mouse cardiac muscle fibers and dynamic contractile features were assessed at maximal (pCa 4.3) and submaximal (pCa 5.5) activations. $McTnT_{R144W}$-reconstituted fibers revealed the following. The speed of crossbridge (XB) recruitment, *b*, decreased significantly at both pCa 4.3 and pCa 5.5; however, the magnitude of decrease was 2-fold greater at submaximal activation. The speed of XB detachment dynamics, *c*, also decreased and was 1.7-fold greater at submaximal activation. However, the XB strain-mediated effects on the recruitment of other XBs (γ) - mediated by allosteric/cooperative mechanisms operating within the thin filament - decreased to a similar extent at both Ca^{2+} activations. Novel findings from our study will be discussed in terms of the $McTnT_{R144W}$-induced effects on the thin filament cooperativity and its associations with slower rates of XB recruitment and detachment kinetics at physiologically relevant Ca^{2+} concentrations.

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Effects of Pseudophosphorylation of Rat Cardiac Troponin T Residue 204 are Differently Affected by α- and β-Myosin Heavy Chain Isoforms

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We tested our hypothesis that α-myosin heavy chain (MHC) and β-MHC differently modulate the functional effects of protein kinase C (PKC)-mediated phosphorylation of rat cardiac troponin T (RcTnT). We generated a chimeric pseudophosphorylated RcTnT in which the threonine 204 was replaced by glutamic acid ($cTnT_{T204E}$) to mimic the PKC-mediated phosphorylation effect. Recombinant proteins were reconstituted into detergent-skinned cardiac muscle fibers from normal rats expressing α-MHC or propylthiouracil-treated rats expressing β-MHC. Steady state measurements revealed that Ca^{2+}-activated maximal tension and the corresponding ATPase activity decreased significantly by ~75% in α-MHC+$cTnT_{T204E}$ fibers, but only by ~33% in β-MHC+$cTnT_{T204E}$ fibers. However, the myofilament Ca^{2+} sensitivity (pCa_{50}) decreased by ~50% in both α-MHC+$cTnT_{T204E}$ and β-MHC+$cTnT_{T204E}$ fibers, suggesting that the greater decrease in maximal tension observed in α-MHC+$cTnT_{T204E}$ fibers cannot be merely attributed to the decrease in Ca^{2+}-mediated activation of thin filaments. Interestingly, the rates of tension redevelopment (k_{tr}), crossbridge (XB) recruitment dynamics (*b*), XB distortion dynamics (*c*), and the tension cost (ATPase rate/tension) decreased only in α-MHC+$cTnT_{T204E}$ fibers. Our results demonstrate that α- and β-MHC isoforms have different impact on

the $cTnT_{T204E}$-mediated effects on cardiac contractile function. Our novel observations have clinical relevance because increased activity of various PKC isoforms and upregulation of β-MHC are observed in failing human hearts.

3890-Pos Board B618

Force-Sarcomere Length Relations in Patients with Thin Filament Myopathy Caused by Mutations in NEB, ACTA1, TPM2 and TPM3

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Mutations in the nebulin gene (NEB), skeletal muscle alpha-actin1 gene (ACTA1), beta-tropomyosin 2 gene (TPM2) and alpha-tropomyosin 3 gene (TPM3) lead to thin filament myopathies, such as nemaline myopathy (NM), congenital fiber type disproportion (CFTD) and cap disease (CAP). A hallmark feature of these myopathies is muscle weakness. Here, we aimed to elucidate the effect of NEB, ACTA1, TPM2 and TPM3 mutations on thin filament length by determining the sarcomere length-dependence of force.

Quadriceps biopsies from NM, CFTD, and CAP patients (n=18) with mutations in the NEB, ACTA1, TPM2 or TPM3 were compared to biopsies from controls (n=3). Using permeabilized muscle fibers, maximal active tension was determined at incremental sarcomere lengths (range 2.0-3.5 μm) to obtain the force-sarcomere length relationship.

The maximal active tension (Fmax (in mN/mm2, mean ± SD)) was significantly lower in biopsies from severe NEB (18 ± 5), mild NEB (76 ± 5), severe ACTA1 (54 ± 13) and severe TPM3 (95 ± 14) patients compared to biopsies of controls (164 ± 17), whereas no significant changes in Fmax were observed in biopsies from mild ACTA1 (139 ± 27), mild TPM2 (1201 ± 8) and mild TPM3 (156 ± 13) patients. The classification of severity is based on the age of onset. No shift in the force-sarcomere length relationship was observed in mild ACTA1, TPM3 and TPM2 patients. Interestingly, in contrast to patients with ACTA1, TPM2 and TPM3 mutations, fiber preparations from both mildly and severly affected NEB patients showed a leftward shift of the force-sarcomere length relationship (a leftward shift of the force-sarcomere length relationship indicates shorter thin filaments).

Our data suggest that mutations in NEB result in the most pronounced changes in thin filament length. Insights in the mechanisms underlying weakness in patients with thin filament mutations are necessary to improve specific treatment strategies.

3891-Pos Board B619

Prolonged Relaxation Kinetics in Distal Arthrogryposis Skeletal Muscle Myofibrils with a MYH3 R672C Mutation

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The mechanisms underlying most forms of Distal Arthrogryposis (DA), a group of congenital contracture syndromes, are unknown. Our previous functional studies from adult individuals with DA caused by the heterozygous embryonic myosin heavy chain mutation, *MYH3* R672C, showed that the time required by DA skinned skeletal myofibers to relax completely after calcium-induced contraction was several-fold longer than controls (Racca *et al*. (2010) *Biophys J* 98:542-3a). Here we measured force and kinetics of activation & relaxation, and compared chemomechanical analysis using myofibrils and myofibers sampled from gastrocnemius muscle from two affected individuals vs. three control (non-DA) individuals. The prolonged relaxation was reflected in isolated myofibrils from DA patients, as 50% relaxation time from maximal activation was 36% longer, and 90% relaxation time was prolonged by 58%. The kinetics of the slow phase of relaxation were significantly slower, in both the rate and duration (DA: $k_{REL,SLOW}$=0.36 ± 0.07s^{-1};$t_{REL,SLOW}$=285 ± 21ms vs. Control: 0.79 ± 0.18s^{-1};199 ± 23ms), implying slower cross-bridge release. Use of ADP prolonged relaxation of control samples to a greater extent than DA preparations, suggesting that slower ADP release from myosin in DA myofibrils may be the mechanism of slower relaxation and cross-bridge release. We also found that both mRNA and protein for this "embryonic" myosin (gene *MYH3*) were present in adult skeletal muscle, such that a small amount of this slower myosin may prolong relaxation. Although the mechanism that leads to the congenital contractures must begin prenatally, these results suggest that *MYH3* R672H also affects ongoing function of adult skeletal muscle. Understanding the mechanism by which myosin mutations affect muscle cell contractility could provide a model for exploring the pathogenesis of more common contractures such as idiopathic clubfoot and facilitate the development of novel therapeutic approaches. **Supported by** F31AR06300(A.R.), 5K23HD057331(A.B.), HD048895(M.B.,M.R.).

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Functional Effects of the β-Myosin Mutation Arg453Cys in Familial Hypertrophic Cardiomyopathy

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In Familial Hypertrophic Cardiomyopathy (FHC) 1/3 of the patients are affected by mutations in the ß-myosin heavy chain (ß-MyHC), the myosin isoform of the ventricle and of slow skeletal muscle fibers in humans. Yet, not much is known about (i) direct effects of specific ß-MyHC-mutations on acto-myosin function, and (ii) how these mutations may trigger development of the FHC-phenotype.

To address these questions, we analyzed the effects of the ß-MyHC-mutation R453C on contractile properties of slow (type I) fibers from the *M. soleus* of a severely affected FHC patient. We found an about 12% higher isometric force, 15% faster rate constant of force redevelopment (k_{tr}), 10% higher isometric ATPase activity and essentially unchanged tension cost. Together with only slightly higher fiber stiffness in rigor, a main part of the increase in isometric force appears to be due to altered cross-bridge cycling kinetics, specifically an increase in f_{app}, the rate constant for the cross-bridge transition into force generating states. Currently we investigate whether increased force generated per myosin head e.g., by a larger y_0-value also contributes.

Relative quantification of mutated vs. wildtype ß-MyHC-mRNA of the heterozygous patient revealed a fraction of about 35% for the R453C-mRNA. Assuming similar abundance of mutated MyHC at the protein level, as we had found in other FHC-mutations, about a third of the ß-myosin heads in the sarcomeres carry the mutation. Thus, force contribution of the mutated myosin head population is about 40% increased. A larger variability in pCa_{50} among individual fibers with mutation R453C vs. control fibers, as we had seen for other FHC-mutations, suggests unequal expression of mutated myosin. We hypothesize that unequal expression of mutated myosin in individual cardiomyocytes causes imbalanced force generation and initiates functional impairment of the myocardium in FHC.

3893-Pos Board B621

The Structure-Function Analysis of Myosin Pseudo-Phosphorylation in Mouse Model of FHC

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Familial hypertrophic cardiomyopathy (FHC) is a disease of the heart caused by autosomal dominant mutations in genes coding for all major sarcomeric proteins, including the myosin regulatory light chain (RLC). In this report, we have explored the rescue strategies to ameliorate the malignant cardiomyopathy phenotype induced by an aspartic acid to valine substitution (D166V) in the RLC. Previous studies on porcine reconstituted preparations showed that the phosphorylation mimic (S15D) in the background of the D166V mutation (S15D-D166V) restored the calcium sensitivity of force and improved V_{max} of myosin ATPase that were largely compromised by the D166V mutation. Transgenic "Rescue Mice" carrying the S15D-D166V mutation have been generated and subjected to structural and functional measurements. Small angle X-ray studies on freshly skinned papillary muscle fibers revealed that a D166V-induced reorganization in cross-bridge mass distribution was partially reversed in Rescue Mice (abnormally increased $I_{1,1}/I_{1,0}$ ratio observed in Tg-D166V fibers returned to the value near Tg-WT). Noteworthy, pseudo-phosphorylation of D166V significantly restored fiber elasticity allowing for changes in the cross-bride mass distribution on stretch. *In vivo* cardiac morphology and function were assessed by non-invasive echocardiography followed by invasive left ventricular pressure-volume measurements (P-V loops). Echocardiography assessment confirmed hypertrophy in Tg-D166V mice showing an increased posterior wall thickness in systole. Invasive hemodynamics showed diastolic and systolic dysfunction in Tg-D166V mice. The end-systolic P-V relationship, a measure of heart contractility, which was largely reduced in Tg-D166V mice was completely ameliorated in Rescue Mice. In addition, the maximum dP/dt - End-Diastolic Volume relation, which was compromised in Tg-D166V was fully reversed in Rescue Mice. In conclusion, our results suggest that pseudo-phosphorylation of myosin RLC can mitigate both the structural and functional abnormalities of the FHC heart. Supported by NIH-HL108343 and HL071778 (DSC).